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Running title: Age-Related Degeneration of Rotator Cuff

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ABSTRACT

Massive tears of the rotator cuff are often associated with progressive and irreversible muscle degeneration due to fibrosis, fatty infiltration, and muscle atrophy. Rotator cuff tears are common in individuals older than 60 years and the repair of these tears ~~are~~ is amongst the most prevalent of orthopaedic procedures. However, most current models of this injury are established in ~~physiologically~~ young animals, which may not accurately recapitulate the clinical condition. In this study we used a murine model of massive rotator cuff tears to evaluate age-related muscle degeneration following chronic injury. The expression of the fibro-adipogenic genes encoding collagen type III and leptin was higher in aged rotator cuff compared to matched injured young tissue at 2 weeks post-injury and development of fibrosis was accelerated in aged mice within 5 days post-injury. Furthermore, synthesis of collagens type I and -III and fat tissue accumulation were significantly higher in injured rotator cuffs of aged mice. Similar frequency of fibro-adipogenic PDGFRβ^+PDGFRα^+ progenitor cells was measured in non-injured rotator cuff of aged and young mice, but PDGFRβ^+PDGFRα^+ cells contributed to significantly larger fibrotic lesions in aged rotator cuffs within 2 weeks post-injury, implying a more prevalent fibrotic environment in the aged injured muscle. Altogether, these findings demonstrate age-dependent differences in rotator cuff response to chronic injury with a more profound fibro-adipogenic change in aged muscles. Clinically, cell therapies for muscular pathologies should not only consider the cell type being transplanted but also the recipient milieu into which these cells are seeded.

Keywords: rotator cuff tear; aging; skeletal muscle; fibrosis; fatty degeneration; fibro-adipogenic progenitor cell

INTRODUCTION

Rotator cuff (RC) tears are increasingly common in individuals older than 60 years and treatment and repair of tears are amongst the most prevalent of orthopaedic procedures.[1] Even with advancements in techniques and procedures for repair, re-tear rates have been shown to range from 11 to 57% depending on factors such as patient age, large tear size and tendon degeneration,[2-5] and muscle atrophy and fatty infiltration in the RC muscles leading to reduced healing potential of the RC.[6] More than half of the population older than 70 years will develop full-thickness RC tears, which can impact quality of life and adversely affects daily functioning.[6, 7] Elderly patients also tend to have diminished healing potential and effectiveness of RC repair due to the aforementioned factors, as well as generally having more comorbidities and a propensity for larger RC tears and more substantial tendon degeneration.[6, 8-11] Thus, outcomes of RC repair surgeries are poorer in this population due in large part to increased re-tear rates and RC healing failure and subsequent loss of strength and decreased function. [6, 8-10]

A positive correlation exists between age and RC tissue degeneration, prevalence of full thickness tears, and tear size, indicated by the number of tendons involved.[12, 13] These data suggest that the increased prevalence of RC pathology with age is a function of persistent RC degeneration over time. Despite the significance of this issue, the age-related pathophysiological mechanisms underlying the degenerative changes in older RC tissue have yet to be elucidated.[14]

Regenerative therapies using stem and progenitor cells may be employed to enhance healing and diminish the effect of fatty infiltration and muscle atrophy following RC tears.[15] Myogenic progenitor cells may not only reduce muscle atrophy but may also foster regeneration of muscle tissue.[16] Murine models have been established for studying the effects of chronic RC tears and subsequent fatty degeneration to gain a better understanding of the pathophysiologic

changes that occur in aging human RC muscles.[17] Additionally, small animal models are being used to study stem cell injection as well as the tissue and gene expression profiles of RC tears in humans.[15, 16] Mice can incur pathological changes post supraspinatus and infraspinatus tissue transection and denervation of the suprascapular nerve (TTDN) similar to those seen in humans following massive RC tears.[18] In this study we compared –RC –remodeling and degeneration, histologically and with respect to -gene expression, between young and old mice following TTDN to determine the validity of using either age group as models for massive RC injury. In mice, senescence starts around 18 months, when the biomarkers of old age are detected. [19] -Accordingly, we used ~~old~~ mice ranging from 18 to 24 months of age, which matches humans ranging from 56 to75 years. We found increased fibrosis and fat accumulation in old non-injured and chronically injured RC muscles in comparison to young RC muscle. Additionally, the frequency of interstitial PDGFRβ^{+}PDGFRα^{+} fibro-adipogenic progenitor cells was similar between non-injured young and old muscle. However, a substantial increase in PDGFRβ^{+}PDGFRα^{+} cells populating fibrotic lesions was measured in old RC within 6 weeks post TTDN.

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METHODS

Mice

PDGFRβ-Cre mice were crossed with mTmG (tdTomato-EGFP) mice. C57/BL6J mice were used as PDGFRβ-Cre mice matched wild type strain. All animal procedures were approved by the local Institutional Animal Care and Use Committee (IACUC). Mice at the age of 3-4 months were considered young and mice older than 18 months were considered old.[19, 20]

Rotator Cuff Injury Model

We induced massive RC tears in old and young mice. We anesthetized the mice with 2% isoflurane and oxygen, administered buprenorphine for analgesia, and sterilely prepared and draped the right shoulder. A 1-cm longitudinal skin incision was made over the right glenohumeral joint to access the deltoid fibers, which were then split directly posterior to the deltoid tuberosity longitudinally to reach the supraspinatus and infraspinatus tendons. These tendons were isolated and sharply detached from their insertions on the greater tuberosity; additionally, the distal 5 mm of each tendon was resected to prevent scar formation to the humerus. Next, the suprascapular nerve was identified through a 5 mm incision in the trapezius musculature anterior to the lateral scapula and cut for the denervation procedure. Lastly, a 5-0 Vicryl (Ethicon, Somerville, NJ, USA) suture was used to close the deltoid muscle and skin.

Histology and Immunohistochemistry

Infraspinatus and supraspinatus muscles were fixed in 4% formalin, embedded in paraffin, sectioned, dehydrated, and stained with hematoxylin and eosin for general tissue structure analysis or picrosirius red for collagen expression according to manufacturer instructions (Abcam, Cambridge, UK). Both muscles, supraspinatus and infraspinatus were always prepared for

117 histology in the same orientation and sectioned in the axial plane and all multiple sections from a
118 single muscle were 5 μm thick. Images were acquired with an Axio Imager 2 light microscope
119 (Zeiss, Oberkochen, Germany). For histological examination, injured young and old cohorts were
120 divided into 3 groups and analyzed at 5 days, 2 weeks and 6 weeks post operation (n = 3 mice per
121 group). Non-injured young and old mice were used as controls (n = at least 3 mice per group). For
122 fluorescence microscopy, frozen sections were fixed with 4% paraformaldehyde, washed 3 times
123 in PBS, immunolabeled with rabbit anti-mouse PDGFRβ and goat anti-mouse PDGFRα overnight
124 at 4°C, washed 3 more times in PBS, and then incubated with Alexa Fluor 647-conjugated, donkey
125 anti-rabbit and Alexa Fluor 405-conjugated, donkey anti-goat secondary antibodies (Abcam).
126 DAPI (4'6-diamino-2-phenylindole dihydrochloride, 1:1000, Molecular Probes, Waltham, MA)
127 was used for nuclei labeling. Images and movies were acquired with the Axio Imager 2 light
128 microscope. For immunohistochemical analysis, injured young and old cohorts were divided into
129 3 groups and analyzed at 5 days, 2 weeks and 6 weeks post operation (n = 3 mice per group). Non-
130 injured young and old mice were used as control (n = 3 mice per group).
131
132 **Quantification of Fibrosis and Adipocytes**
133 Following picrosirius red staining as described above, fibrosis was quantified in injured and non-
134 injured, young and old tissue sections by red pixel intensity measurement by Photoshop and the
135 fraction of fibrosis was calculated by dividing the number of red pixels by the entire number of
136 pixels per area. Adipocytes were counted in hematoxylin and eosin stained RC sections for
137 quantification of fat content. Based on our observations that RC degeneration spreads laterally,
138 images were not taken randomly; instead, the whole area of each section was screened and all
139 fibrotic or adipogenic regions were imaged at the same magnification of x200. Therefore, the

number of images per section varied based on the relative size of the fibrotic or the adipogenic area.

RNA Extraction and Reverse Transcription PCR

The infraspinatus and supraspinatus muscle tissues were immediately frozen and stored at -80°C following harvest. RNA was isolated from muscle tissue using ADD KIT and its concentrations were measured with NanoDrop (Thermo Fisher Scientific, Waltham, MA). The RNA was then reverse transcribed to complementary DNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA) and the iCycler thermal cycler (BioRad). We ran the PCRs using 130-200 ng of RNA under the following cycling conditions: 5 min at 25°C for priming, followed by 20 min at 46°C for reverse transcription, and finally, 1 min at 95°C for reverse transcriptase inactivation. We quantified the complementary DNA using Absolute SYBR Green Low ROX qPCR Mix (Life Technologies, Carlsbad, CA) and the ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following cycling conditions: 15 min at 95°C for enzyme activation, followed by 40 cycles of amplification (15 s at 95°C, 30 se at 60°C, and 30 s at 72°C). Gene expression profiles were determined by analyzing quantitative RT-PCR data of collagen and leptin genes by calculating the fold change ($2^{-\Delta\Delta Ct}$) in gene expression compared to the expression of the housekeeping gene GAPDH. Primer sequences (Integrated DNA Technologies, Coralville, IA) used for RT-PCR: GAPDH-F CCTGGAGAAACCTGCCAAGTATG, GAPDH-R AGAGTGGGAGTTGCTGTTGAAGTC; leptin-F TCCTGTGGCTTTGGTCCTATC, leptin-R ATACCGACTGCGTGTGTGAA; Col3A1-F AGGCTGAAGGAAACAGCAAA, Col3A1-R TAGTCTCATTGCCTTGCGTG. For RT-PCR analysis, cohorts were divided into 3 groups and analyzed at 5 days, 2 weeks and 6 weeks post operation. Non-injured young and old mice were used as controls.

Flow Cytometry

Non-injured young (n=3, 3-4 months) and old (n=3, 18-20 months) RC muscles were excised, mechanically minced, and dissociated using 0.5 mg/mL collagenase II and dispase (Sigma, St Louis, MO, USA) in Dulbecco's modified Eagle~~'s~~ medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) for 30 min at 37°C on a shaker. Freshly isolated cells were washed in PBS, centrifuged, and labeled with PE/Cy7-conjugated anti-mouse PDGFRα and APC-conjugated anti-mouse PDGFRβ (eBioscience, San Diego, CA) according to the manufacturer's instructions. We used the LSR II and FACSDiva flow cytometers (BD Biosciences, San Jose, CA) for subsequent analyses.

Statistical Analysis

All data are presented as mean+SEM. Single factor ANOVA was used to compare mean values among study groups (Excel 2010) and two-way ANOVA was used to analyze effects of injury and age on gene expression. We controlled the family-wise error rate by using a Bonferroni correction. For all analyses, a P value of ≤ 0.01 was considered statistically significant.

RESULTS

Irreversible Nerve and Tendon Transection Induces Degeneration of Young and Old Murine RC

To evaluate the changes in muscle tissue morphology following induction of chronic muscle injury by nerve and tendon transection (TTDN), RC muscle was harvested from young (2-4 months) and old mice (20-24 months) at early (5 days), intermediate (2 weeks) and late (6 weeks) stages of muscle remodeling post-injury. Histological examination of hematoxylin and eosin stained sections of non-injured and injured RC from young and old mice revealed that, in comparison to the normal appearance of healthy young (Fig. 1A) and old muscle (Fig. 1E), TTDN induced a robust increase in muscle cellularity accompanied by myofiber necrosis within 5 days regardless of mouse age (Fig. 1B and F). At 2 weeks post-TTDN, myocyte regeneration was observed with an increase in myofibers with central nuclei, a hallmark of the regenerative process. Additionally, the accumulation of fat cells was seen in both young (Fig. 1C) and old (Fig. 1G) RC, and this fatty infiltration was greater at this time point relative to the 5-day tissue samples. Six weeks after TTDN, histology of both the young (Fig. 1D) and old (Fig. 1H) RC tissues revealed fibro-adipogenic changes, which were more pronounced in the old RC tissue.

Development of Fibrosis is Accelerated in Chronically Injured RC of Old Mice

Development of fibrosis is defined by increased deposition of collagens type I and III and was evaluated by quantification of red pixel intensity after picrosirius red staining of RC muscle sections. Progressive increase in collagen content was measured in both old and young injured RC within 6 weeks post-TTDN (Fig. 2I). Quantification of picrosirius red staining revealed that collagen content was higher in non-injured old RC (n=3, 20-21 months) compared to non-injured

young RC (n=4, 3-4 months, $p<0.005$), and that collagen accumulation in fibrotic lesions was significantly greater in old injured RC at 5 days (n=3, 22 months, $p<0.001$) and 2 weeks (n=3, 20-22 months, $p<0.005$) post-TTDN in comparison to young RC (Fig. 2I)), indicating accelerated fibrogenesis in chronically injured old RC muscle. The 6-week post-TTDN young (n=3, 3-4 months) and old (n=3, 20-22 months) tissues had the most pronounced fibrotic change overall compared to earlier time points after injury of each age group (Fig. 2I, $p<0.01$), the old RC tissue demonstrating more collagenous infiltration than the young RC. There was no significant interaction between mouse age and injury on collagen synthesis (two-way ANOVA, $p<0.01$).

Quantitative PCR of collagen III expression coincided with the observed increase in fibrosis that was quantified for non-injured old RC muscle ($p<0.005$) as well as over time for both the young and old RC tissues following TTDN (Fig. 2J). In both old and young RC, expression of collagen III was induced by injury within 5 days (n=5, 20-22 months, $p<0.01$), escalated at 2 weeks post-injury (n=3, 20-22 months) and significantly declined at 6 weeks post-TTDN (n=5, 20-25 months, $p<0.005$), when both the young and old tissues had already become progressively more fibrotic. Synthesis of collagen I peaked at 5 days post injury in young injured RC and lasted longer in old RC peaking at 2 weeks after injury in old RC (Fig. 2K). The relative expression pattern of matrix metallopeptidase 2 (MMP2) was similar to that of collagen I (Fig. 2L) with the expression of both genes declining at 6 weeks post-TTDN (Fig. 2K-L). A two-way ANOVA revealed significant interaction between mouse age and injury on gene expression levels of collagen I ($F_{3,26}=7.6$, $p<0.01$) and MMP-2 ($F_{3,24}=6.99$, $p<0.01$).

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Greater Fat Tissue Accumulation is Observed in Chronically-Iinjured Old RC

To assess age-related differences in muscle tissue fatty degeneration following massive RC tear, we performed TTDN on old and young mice and analyzed post-injury adipogenesis at various time points. Histological analysis revealed that while adipocytes were rarely detected in non-injured young (n=4, 3-4 months, Fig. 3A) and old (n=3, 20-21 months, Fig. 3E) RCs, small adipocyte clusters were seen in injured young and old RC within 5 days (n=5, Figs. 3B and 3F) and 2 weeks (n=3) post-TTDN. Robust increase in fat tissue accumulation was observed in both young and old RC tissues at 2 weeks (Figs. 3C and 3G, $p<0.001$) and 6 weeks following TTDN (Fig. 3D and 3H, $p<0.001$). No significant increase was found in adipocyte numbers between injured young and old RC at 5 days post-TTDN (Fig. 3I). However, considerably more adipocytes were counted in old injured RC (n=3, 3-4 months) in comparison to young RC at 2 and 6 weeks (n=4, 20-22 months) post-TTDN (Fig. 3I, $p<0.01$), implying that the degenerated microenvironment of old RC promotes accelerated growth of adipose tissue at late stages of chronic injury. Two-way ANOVA revealed significant interaction between mouse age and injury on adipocyte count ($F_{3,152}=6.28$, $p<0.01$).

Commented [PB3]: These are young

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Gene expression of leptin (Fig. 3J), a hormone that is released from fat cells, was progressively elevated in both young and old degenerating RC (Fig. 3J) and coincided with the increase in the numbers of adipocytes that were quantified over time for both the young and old RC tissues following TTDN (Fig. 3I). At 6 weeks post-TTDN, old RC demonstrated the highest levels of leptin gene expression (Fig. 3J), corresponding with higher adipocyte number in old RC in comparison to young RC at the same time point post-TTDN (Fig. 3I). Expression of adiponectin was overall higher in old injured RC at 5 days and 2 weeks post-TTDN compared to matched young injured RC (Fig. 3K) and significantly declined in old RC within 6 weeks after induction of

injury (Fig. 3K). These findings demonstrate that older mice develop more post-TTDN fatty degeneration of RC muscle tissue than young mice within 2 weeks. There was no significant interaction between mouse age and injury in the expression levels of the tested adipogenic genes (two-way ANOVA, $p<0.01$).

PDGFRβ$^+$PDGFRα$^+$ Fibro-adipogenic Progenitor Cells Have the Same Frequency in Non-injured Young and Old RC Muscle Tissue

The acceleration and increase in fibro-adipogenic response in old injured RC can be attributed to differences in the frequency of fibro-adipogenic progenitor cells between young and old RC. We have previously demonstrated that PDGFRβ$^+$PDGFRα$^+$ progenitor cells contribute to tissue fibro-adipogenesis after injury [12] and therefore we used flow cytometry analysis to determine the frequency of PDGFRβ$^+$PDGFRα$^+$ fibro-adipogenic precursors in RC of non-injured young (n=4, 3-4 months old) and old (n=3, 18-20 months old) mice. All PDGFRα$^+$ cells co-expressed PDGFRβ (Fig. 4A) and there was no significant difference in the frequency of fibro-adipogenic PDGFRβ$^+$PDGFRα$^+$ cells between non-injured young and old RC tissue (Fig. 4B and 4C), implying that greater degeneration of injured old RC is attributable to the microenvironment, which would induce more active proliferation and differentiation of these fibro-adipogenic cells. Alternatively, or concomitantly, the intrinsic fibro-adipogenic potential of these PDGFRβ$^+$PDGFRα$^+$ cells might increase with age (higher collagen production on a per-cell basis, for instance).

To test the former hypothesis, we performed multi-color immunofluorescence staining of PDGFRβ and PDGFRα in non-injured and injured young and old RC sections (Fig. 4D) and

quantified ~~the~~ pixels ~~color~~ representative of PDGFRβ expression, PDGFRα expression or PDGFRβ and PDGFRα co-expression. At each tested time point post-TTDN, a similar pixel fraction was measured for PDGFRβ, PDGFRα or co-expression of PDGFRβ and PDGFRα, demonstrating that the fibrotic lesions are mainly populated by $PDGFR\beta^{+}PDGFR\alpha^{+}$ cells (Fig. 4E). The dynamic frequency of this subset was measured throughout the post-injury remodeling process of the RC and was shown to have a similar trend in both young and old RC at 5 days and 6 weeks post-TTDN but not at 2 weeks post-TTDN (Fig. 4E). The frequency of $PDGFR\beta^{+}PDGFR\alpha^{+}$ cells was increased within 5 days ($p<0.01$) as well as 2 and 6 weeks post-TTDN ($p<0.001$ and $p<0.00001$ respectively) in both young and old RC in comparison to non-injured muscle. However, a transient decrease in the frequency of $PDGFR\beta^{+}PDGFR\alpha^{+}$ cells was measured only in young injured RC at 2 weeks post-TTDN (Fig. 4E). At 2 and 6 weeks post-TTDN, immuno-staining for PDGFRβ and PDGFRα illustrated larger fibrotic lesions in old RC tissue compared to young RC tissue post-injury (Fig. 4D), coinciding with greater measured color fraction of $PDGFR\beta^{+}PDGFR\alpha^{+}$ cells in old degenerated RC at 2 and 6 weeks post-injury (Fig. 4E, $p<0.01$ and $p<0.0001$ respectively) and thus implying a more robust pro-fibrotic environmental cues in old, injured RC muscle, which drives higher proliferation of fibro-adipogenic progenitor cells.

DISCUSSION

Rotator cuff tears are one of the most common musculoskeletal injuries and a substantial source of morbidity in elderly patients. Massive RC tears, in particular, are associated with muscle atrophy, fatty degeneration, and fibrosis. These degenerative processes interfere with tissue healing and are associated with poor surgical outcomes. In the present investigation, we found that

an increase in fibrosis and fat accumulation are associated with RC aging and substantially increased following tendon transection and denervation, in comparison to young RC. However, we measured a similar frequency of PDGFRβ^{+}PDGFRα^{+} pro-fibrotic cells in non-injured young and old RC, which indicates that the formation of larger fibrotic lesions in old injured RC can be attributed to microenvironmental cues, mediating increased expansion and/or differentiation of PDGFRβ^{+}PDGFRα^{+} fibrogenic cells. Alternatively, or in parallel, the intrinsic fibro-adipogenic potential of these PDGFRβ^{+}PDGFRα^{+} cells might increase with age. Supporting the notion that young fibro-adipogenic progenitors differ from their aged counterparts, it was recently reported that loss of secretion of WISP-1 from aged muscle-residing fibro-adipogenic progenitor cells impairs efficient muscle regeneration, but can be rescued by administration of young muscle-derived fibro-adipogenic progenitor cells.[21]

We found that older mice had greater amounts of fibrosis in RC muscle than younger mice, even prior to injury. In accordance, increase in collagen deposition has been observed in other muscle types of aged rats and mice. A two-fold increase in fibrotic lesions and collagen deposition was measured in hind limb soleus and extensor digitorum longus muscles of 2-year-old rats[22] as well as in the tibialis anterior muscles of 28-30-month-old mice[23] in comparison to matched young adult animals. Aging induced fibrosis was shown to relate to loss of muscle neuronal nitric oxide synthase that is associated with an increase in intramuscular leukocytes, especially M2a macrophages that can promote muscle fibrosis via arginase-mediated metabolism.[24] Aging of the bone marrow leads to a shift in myeloid cells in muscle toward the M2a phenotype that occurs independently of muscle age. This shift in macrophage phenotype can further promote muscle fibrosis during aging.[24]

Likewise, human muscles exhibit age-related increase in fat and connective tissue for arm and foot flexors as well as arm extensors,[25] reductions in the cross-sectional area of the quadriceps and hamstrings muscles of elderly men (65–77 years old), and concomitant increases in non-muscle tissue.[26]

We observed a transient progressive increase in collagen deposition within 2 weeks post injury followed by a decrease at 6 weeks post injury. These changes were more prominent in old injured RC and corresponded with increased numbers of fibrogenic PDGFRβ^{+}PDGFRα^{+} cells populating the fibrotic lesions of old injured RC. Several factors have been identified as modulators of collagen synthesis: while TGFβ1, PDGF BB, endothelin 1, angiotensin II and IL-1 stimulate collagen synthesis bFGF, NO, INFγ and TGFα inhibit its production.[27] Possibly, differences in the levels of secreted factors between old and young injured RC are responsible for the more drastic changes in collagen production that are observed in old injured RC.

As opposed to the decline in collagen expression within 6 weeks post injury, the expression of adipogenic leptin was continuously increasing post injury with significantly higher expression in old RC at 6 weeks post TTDN. The leptin hormone is synthesized and secreted primarily by adipocytes and is present in serum in direct proportion to the amount of adipose tissue.[28] Accordingly, increased numbers of adipocytes were quantified over time following ~~induction of~~ injury, ~~with~~ the highest count of adipocytes and, in correlation, highest ~~measured~~ leptin expression being measured in old injured RC at 6 weeks post injury.

Commented [PB5]: This sentence is sort of difficult to understand in its original version

We have recently demonstrated that co-expression of PDGFRβ and PDGFRα on a subset of muscle residing cells defines pro-fibrotic and scar residing fibrotic cells that directly contribute to the formation of fibrotic lesions in the chronically injured murine RC.[29] Our present findings demonstrate age-dependent differences in RC response to injury with enhanced contribution of

fibro-adipogenic PDGFRβ^{+}PDGFRα^{+} cells to muscle degeneration in aged mice. This can be explained in part by the results of studies assessing molecular and cellular changes within aged muscle that revealed several homeostatic perturbations, including decreased Notch signaling and increased activation of TGFβ signaling[30] that also mediates proliferation of myofibroblasts.[31]

Previous reports indicate that the type of experimental RC injury affects the rate of adipogenesis. Only when neurotomy was combined with tenotomy did rats develop severe fat accumulation similar to that seen in human patients.[17] Adipocyte infiltration in the supraspinatus muscle of the RC following tenotomy was observed in both adult and aged rats but was reported as not significantly different between young and old rats.[32] In another study, the infraspinatus was analyzed instead of the supraspinatus and tenectomy resulted in mild adipogenesis that appeared greater in aged rats compared to young rats.[33] Altogether, these findings suggest that the extent of adipogenesis differs between the supraspinatus and the infraspinatus and that the statistically significant contribution to increased adipogenesis that we have measured can be attributed to the infraspinatus. Still, a comprehensive statistical analysis should be performed to validate this assumption using a combined nerve and tendon RC chronic injury. However, there is also a difference between young and old rats in the expression of the stem cell regulatory proteins MyoD and Myf5, that facilitate the differentiation of stem cells into muscle cells.[34] Importantly, though, this study was performed in healthy aged rats as opposed to those with induced RC injury.

Altogether, these findings indicate that aged mice have a greater fibro-adipogenic response to massive RC tears. Future studies utilizing TTDN should use old mice to more accurately recapitulate the human condition. Clinically, cell-based therapies for muscular disease should consider not just the cells to be transplanted, but also the host milieu into which the cells will differentiate and grow.

Limitations Some limitations of this study are inherent in the model itself. Induction of an acute RC injury in a mouse may not be entirely representative of the chronic human clinical condition, and the shoulder joint being weight-bearing in rodents but not in humans must be taken into consideration. These differences may contribute to slightly different injury profiles and environmental milieux. In mice, neurotomy is essential for the induction of severe fat accumulation similar to that seen in human patients.[17] However, absence of functional nerve supply in this model would likely disrupt the evaluation of cell-based therapies aimed at regenerating functional human muscle. Additionally, to the best of our knowledge, there is no standard that currently exists for comparing cellular and genetic profiles in young and old mice and extrapolating these differences to humans. How degenerative changes in tissues seen between young and aged rodents translate to those seen over time in humans is not known. However, uncertainty about such cross-applicability exists for all research performed with animal models as proxies for clinical conditions in humans.

ACKNOWLEDGMENTS

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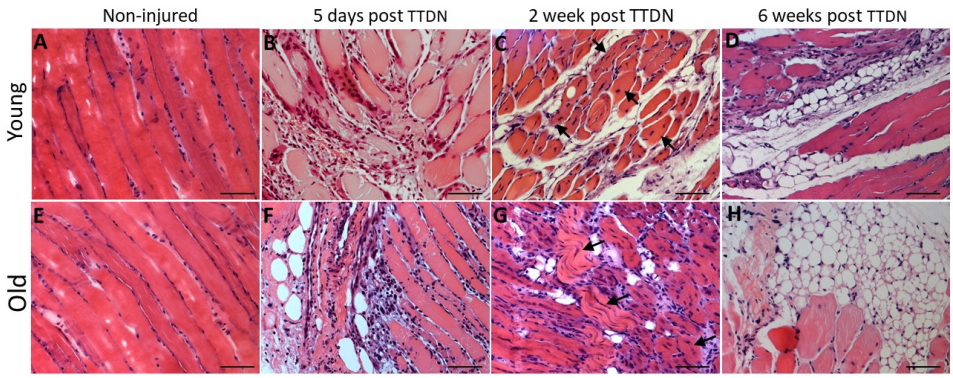
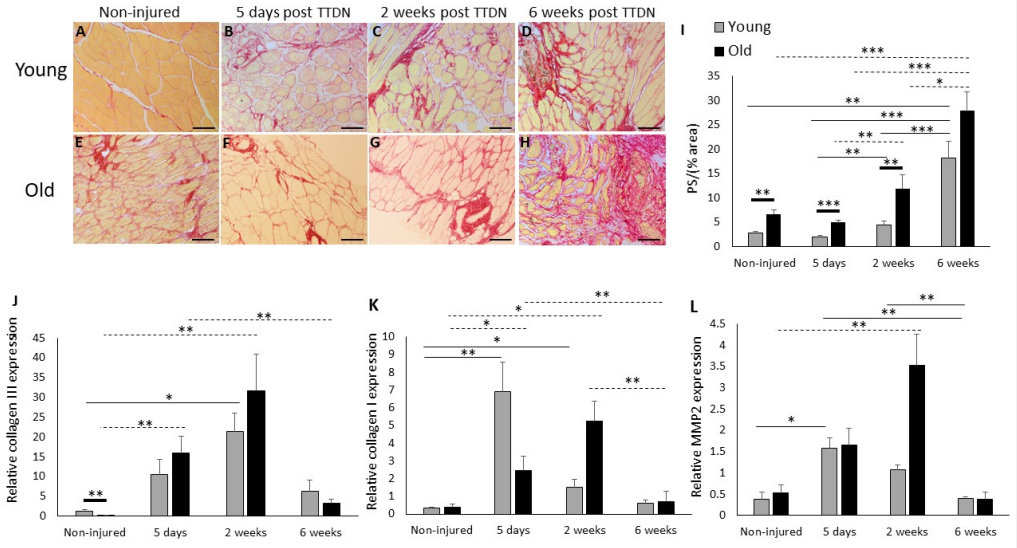


Figure 1. Representative hematoxylin-eosin stained sections demonstrating progressive fibro-adipogenic changes over time in mouse RC following tenotomy and denervation (TTDN). (A-D) Young (3-4 months) and (E-H) old (20-24 months) mouse muscle. (A and E) Normal muscle architecture of healthy, non-injured young (A) and old (E) mouse RC. (B and F) Increased cellularity and necrotic myofibers are seen at five days post-TTDN. (C and G) Myofiber regeneration is indicated by the centrally located nuclei within the myofibers (C and G; arrows) at 2-weeks post-TTDN. (D and H) Increased fibro-adipogenic change is seen in the older cohort relative to the younger cohort at 6 weeks post-TTDN. Scale bar: 50μm.



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408 **Figure 2.** Picrosirius red stain for histological visualization and quantification of collagen
409 deposition in RC tissue sections of young and old mice following TTDN. Collagenous tissue is
410 stained red and is visible between myofibers. (A-D) Young and (E-H) old muscle harvested from
411 non-injured mice (A and E) or at the indicated time points post-TTDN (B-G and F-H). (I) Pixel
412 fraction quantification of red collagen staining in young and old non-injured RC and at the
413 indicated time points post TTDN. (J-L) Relative expression of collagen III (J) collagen I (K) and
414 MMP-2 (L). expression. *$p = 0.01$; **$p = 0.005$; ***$p = 0.001$. n = at least 3 mice per group. Solid
415 line represents comparisons between young RC tissue; Dashed line represents comparisons
416 between old RC tissue; Thick line represents comparisons between young and old RC tissues.
417 Scale bar: 50μm

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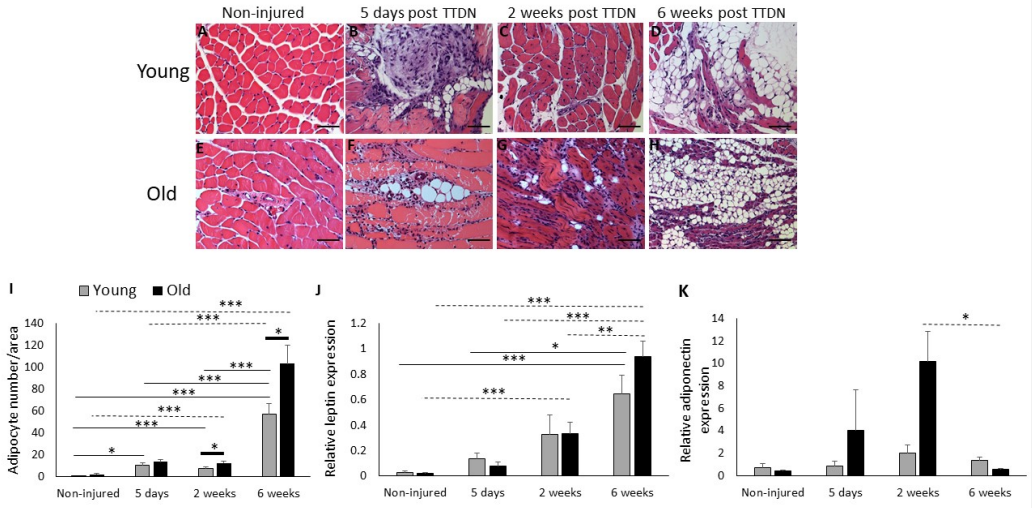

Figure 3. Fat tissue accumulation in young and old RC post-TTDN. Hematoxylin-eosin stained RC sections of (A-D) young and (E-H) old mice. Adipocyte accumulation in young (A-D) and old (E-H) RC harvested from non-injured mice (A and E) or at the indicated time points post-TTDN (B-G and F-H). (A and E) Normal muscle architecture of healthy, non-injured young (A) and old (E) mouse RC. (I) Quantification of adipocytes in young and old non-injured RC and at the indicated time points post TTDN. (J-K) Relative expression of leptin (J) and adiponectin (K). *p = 0.01; **p = 0.005; ***p = 0.001. n= at least 3 mice per group. Solid line represents comparisons between young RC tissue; Dashed line represents comparisons between old RC tissue; Thick line represents comparisons between young and old RC tissues. Scale bar: 50μm.

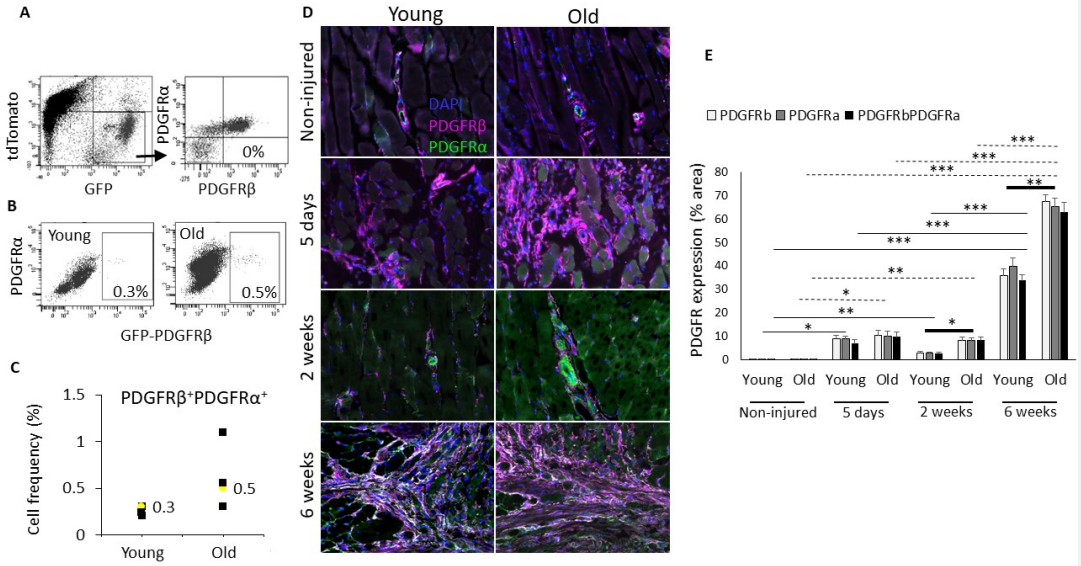

Figure 4. Quantification of PDGFR expression in chronically degenerating RC. (A) Representative flow cytometry dot plots of PDGFRβ+/PDGFRα+ cell subsets. (B-C) Frequency of PDGFRβ^{+}PDGFRα^{+} cells in non-injured young and old RC. (D) Representative images of PDGFRβ (pink) and PDGFRα (green) immuno-staining of non-injured RC and at the indicated time points post-TTDN. Co-localization of PDGFRβ and PDGFRα is seen in light green and white. (E) Quantification of pink (PDGFRβ expression), green (PDGFRα expression) and light green/white (PDGFRβ and PDGFRα co-expression) pixel fraction per imaged area of stained RC sections. No significant difference was observed between PDGFRβ and PDGFRα pixel fraction. *p = 0.01; **p = 0.001; ***p = 0.00001; n =3 mice per group. Solid line represents comparisons between young PDGFRβ^{+}PDGFRα^{+}, dashed line represents comparisons between old PDGFRβ^{+}PDGFRα^{+}, thick line represents comparisons between old and young PDGFRβ^{+}PDGFRα^{+}.

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